# The Effects of Light at Night on Lymphatic Clock Gene Expression in Siberian Hamsters *(Phodopus sungorus)*

an Undergraduate Honors Research Thesis

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by

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**Abstract**

Exposure to light at night can interfere with photoperiodic changes in physiology and behavior in small photoperiodic rodents. Previous studies have shown a balance between immune and reproductive function is required to maximize survival capabilities. Longer summer days are indicative of greater reproductive function, while the shorter, winter-like days yield an increase in immune function to bolster winter survival capabilities. In Siberian hamsters (*Phodopus sungorus)*, dim light at night alters the development of the short-day, winter-like phenotype and blocks enhanced immune function. The circadian and immune systems are tightly linked, but clock gene expression in immune organs under the influence of different photoperiods is not well documented. The goal of this study was to study the photoperiodic clock gene responses in Siberian hamsters, with special attention paid to the influence of dim light at night. Animals were exposed to dim light at night under different photoperiods and clock genes were assayed in the lymph nodes and spleen to map clock gene expression with respect to photoperiod. Alterations in gene expression due to dim light at night may be indicative of humans causing ecological changes through light production and pollution.

**Introduction**

Darwinian fitness is typically considered by scientists to be a measure of reproductive and survival capabilities. Individuals best able to survive are the most likely to reproduce to yield a successful lineage. At times, reproductive capabilities must be balanced with survival priorities, especially in non-tropical environments that yield significant changes between summer and winter seasons. Previous studies have shown proper immune function to be an avenue for survival.[1]

Full investment in both the reproductive and immune systems is energetically costly. Natural selection has not favored energetic investment in both the reproductive and immune systems simultaneously, but rather a selective energetic investment driven by the environment.[2] Most environmental factors are not constant. Weather, temperature, rainfall, barometric pressure, and food availability vary greatly—perhaps from day to day. The photoperiod (i.e., day length) is the most reliable environmental cue for the time of year. Animals can detect the time of year by using the photoperiod and by detecting an increase or decrease in day lengths.[3] The longer, warmer days of summer favor increased reproductive function, while the shorter, colder days of winter favor greater immune function for maximal fitness. In the longer days of spring and summer, resources are abundant and optimal for successful rearing of offspring.[4] Conversely, during the shorter days of autumn and winter, many species bolster immune defenses to increase chances of survival to the next breeding season.[5]

Physiological changes accompany annual photoperiod changes, but these alterations require a great deal of time and energetic investment. Previous studies have shown that neuroendocrine adjustments precede other physiological changes, signaling for the interplay between reproductive and immune function.[6]

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone that has a great effect on circadian rhythms, including the sleep-wake cycle. Melatonin is directly secreted into the blood and cerebrospinal fluid as an endogenous sign of darkness.[7] Light information reaches the pineal gland through a polysynaptic pathway beginning in the retina. Intrinsically photosensitive retinal ganglion cells (ipRGCs) detect light information and feed it into axons traveling in the retinohypothalamic tract, sending information to the suprachiasmatic nucleus (SCN), located within the hypothalamus.[8] The SCN conveys information about the light-dark environment to the

pineal gland via sympathetic postganglionic noradrenergic projections to modulate melatonin secretion.[9]

Changes in day length coincide with alterations in the duration of melatonin secretion. Melatonin communicates circadian and photoperiodic information to peripheral tissues.[10] Removal of the pineal gland has been shown to block photoperiod induced phenotypic changes.[11] Similarly, introduction of exogenous melatonin in pinealectomized animals was enough to elicit a phenotypic response, suggesting that melatonin alone is sufficient to elicit a photoperiodic response.[12] A pineal-independent photoperiodic pathway may exist, but it is not well documented at this time in mammals.[13]

Previous studies suggest that melatonin signaling modulates seasonal immune function variation.[14] The first connection between the immune system and melatonin was made when pinealectomized rats displayed reduced immune function.[15] Furthermore, artificially induced immunosuppression was reversed with the introduction of melatonin.[16] Short-day melatonin levels may facilitate immune recovery by buffering the inflammatory response. Short photoperiods have been shown to reduce fever, hypothalamic cytokine expression, and 'sickness behavior' when artificially induced by an endotoxin, lipopolysaccharide (LPS), in Siberian hamsters (*Phodopus sungorus*).[17] In general, melatonin is an immune-enhancing hormone in small, non-tropical rodents and its effect is most greatly seen in short-day secretion patterns.[18]

Melatonin is not alone in its ability to modulate immune function. Interactions between circulating sex steroids, gonadotropins, and melatonin occur to modulate photoperiodic changes in immune function. Sex steroids appear to have a secondary effect in response to changes in photoperiod, and their photoperiodic response contributing to immune changes is species

dependent.[19] When separate from photoperiod, sex steroids appear to have an important role in immune system modulation.

Non-tropical rodents are sensitive to photoperiodic trends, turning off reproduction and bolstering immune function in presence of short photoperiod to improve odds of winter survival. In small mammals, the reproductive system appears to measure seasonal time by comparing the ambient photoperiod to the one preceding it.[20] Conversely, immune function appears to be modulated by the absolute photoperiod, rather than change in photoperiod.[21] In Siberian hamsters, dim light at night (dLAN) alters the development of the typical short-day (winter-like) phenotype (denoted by light coat color, lower body mass, and lower reproductive organ mass) and blocks enhanced immune function.[22] The immune and circadian systems are tightly linked, but relative circadian clock gene expression and their influence on inflammation in peripheral tissues are not well documented.

*Per1* and *Bmal1* are two core clock genes in the circadian system. Both genes and their protein products are involved in a negative feedback loop. The *Bmal1* protein product activates the *Per1* gene; the *Per1* protein product then goes on to inhibit its own transcription.[23] As such, an antiphasic relationship has been observed in circadian rhythms of *Per1* and *Bmal1* expression in rats.[23] Other circadian clock genes involved in similar regulated processes include the *Per2* and *Clock* genes.

The SCN acts as a ‘master clock’, interpreting signals from external lighting environments to gene expression, while peripheral immune tissues mimic clock gene expression in the SCN.[23] Previous studies have shown circadian clocks in peripheral immune tissues regulate the rhythm of inflammatory responses.[24] Conversely, disruption of circadian clocks

deregulates immune responses, suggesting a tie between proper immune response and proper circadian functioning.[25]

Previous studies have shown the introduction of artificial light at night interrupts the typical synthesis of pineal melatonin; light as low as 1 lux is sufficient to suppress pineal melatonin in Syrian hamsters.[26] Given the importance of appropriate synchronized circadian rhythms to the environmental light-dark cycle, these data suggest dim light at night could negatively affect immune responses by altering circadian rhythms in clock gene expression in genes like *Per1* and *Bmal1*. Because melatonin is only secreted at night, the duration of elevated melatonin secretion encodes night length information to peripheral cells that do not receive direct environmental light information via changes in clock gene expression.

This study hypothesizes that dim light at night (dLAN) interferes with short day enhancement of immune function by altering clock gene expression centrally and in peripheral immune cells due to reduced melatonin secretion. If true, hamsters placed in a 'short day, dim-light-at-night' (SDdim) condition will display impaired immune function. Ultimately, light exposure at night could alter the rhythm of clock gene expression in the SCN, peripheral leukocytes, and lymphatic tissue. Nightly dim light exposure may disrupt the circadian rhythm of immune response to inflammation and trafficking from blood to lymphatics.

## Methods

### *Condition Exposure and Tissue Collection*

One hundred and ninety-three (193) adult male Siberian hamsters (*Phodopus sungorus*) from Dr. Randy J. Nelson's breeding colony were used in this study. Animals were bred and maintained under long day (16:8 h light/dark cycle) conditions before group assignment.

Animals were randomly assigned to one of the following experimental groups in a full factorial design: (1) a standard long day (LD) 16:8 h light/dark cycle (150 lux/0 lux), (2) a standard short day (SD) 8:16 h light/dark cycle (150 lux/0 lux), (3) a long day with dim light at night (LDdim) 16:8 h light/dark cycle (150 lux/5 lux), or (4) a short day with dim light at night (SDdim) 8:16 h light/dark cycle (150 lux/5 lux).

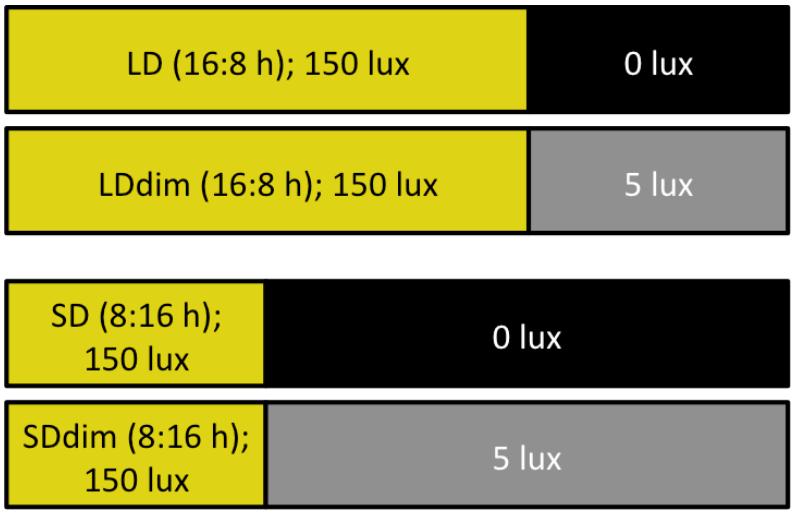


*Fig. 1.* Light Construct for Experimental Groups

Animals were ear punched and were group housed (2-5/cage) in polypropylene cages (27.8 x 7.5 x 13 cm) in light-cycle controlled cabinets with constant temperature and humidity of 21 ± 4 °C and 50 ± 10%, respectively. Daytime light was provided by compact fluorescent bulbs (General Electric, F8T5CW F8 T5 Cool White 12" Bulb), while dim light at night (dLAN) was provided by broad spectrum white LEDs. Light emission was determined and adjusted to experiment specification by a light meter. Light exposure equivalency was ensured throughout the entire cabinet. Each group was maintained according to assignment inside lightproof, ventilated cabinets for 10 weeks. Animals had *ad libitium* access to food (Harlan Teklad 8640 rodent diet; Indianapolis, IN) and filtered tap water. Body mass and pelage scores (integers from

1 to 4; 1 = darkest pelage, 4 = lightest pelage) were observed weekly to assess photoperiodic responsiveness.

*Tissue Collection*

Following light cycle exposure for ten weeks, hamsters were euthanized 'around the clock' at four-hour intervals—0500, 0900, 1300, 1700, 2100, and 0100. Animals were maintained in lighting conditions and individually brought into the procedure room. Each animal was deeply anesthetized with 5% isoflurane via inhalation and a final body mass and pelage score was recorded for each animal. Hamsters were rapidly decapitated and a blood sample was taken from the trunk. The brain, half of the spleen and the left inguinal lymph node of each animal were extracted, placed in RNAlater (Qiagen) reagent, and stored at -80 °C for later analysis. The other lymph node and half of the spleen were placed in HBSS solution (Life Technologies, Thermo Fisher Scientific Inc.) for subsequent cell culturing. Reproductive tissues (testes, epididymides, seminal vesicles, and gonadal fat pads) were dissected and weighed to analyze photoperiodic reproductive system responses.

*RNA Extraction and cDNA Synthesis*

Spleen and lymph samples were maintained at -80 °C for one week before RNA extraction. Lymph node samples were pooled in groups (from animals in the same experimental group and euthanasia time point) to facilitate greater yield in RNA extraction from samples. The pooled lymph samples were homogenized (Nippi, PowerMasher II) and RNA was extracted with TRIzol reagent (Life Technologies, Thermo Fisher Scientific Inc.) according to the manufacturer's extraction protocol. The extracted RNA pellet was resuspended in 30 µL RNase-

free water. A spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific Inc.) was used to determine RNA quantity and quality. Samples selected for cDNA synthesis and subsequent qPCR analysis had RNA yields above 200 ng/μL and 260/280 and 260/230 ratios between 1.8 and 2.3 to ensure high quantity and quality. RNA was treated with DNase I (Invitrogen) to prevent amplification of residual genomic DNA. For each sample, 0.1 μg of RNA was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase enzyme (Promega,WI) according to the manufacturer's protocol, then diluted 1:10 for qPCR analysis.

*Quantitative Polymerase Chain Reaction (qPCR)*

*Per1* and *Bmal1* were individually compared to 18S rRNA expression for relative quantification. The polymerase chain reaction was performed using a 7500 Fast Real-Time PCR System (Life Technologies, Thermo Fisher Scientific Inc.) with SYBR Green chemistry. For real-time PCR analysis, 1% of the cDNA was used at a final concentration of 1x Power SYBR Green PCR Master Mix (Life Technologies, Thermo Fisher Scientific Inc.) and 0.05 μM of each primer. Designed[27] forward and reverse primer sequences for *Per1* and *Bmal1* in *Phodopus sungorus* were utilized in the PCR reaction. Forward and reverse primer sequences were 5'-GGT TCG CAG CAG CCA AA-3' and 5'-TGA GGA GTC GAT GCT ACC AAA G-3', respectively, for *Per1*, and 5'-GGC AGC GAT GGC TGT CA-3', and 5'-TCC ACC CAG GCC TGC AT-3', respectively, for *Bmal1*. The PCR cycling conditions used were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Samples were run in duplicate within 96-well PCR plates (Life Technologies, Thermo Fisher Scientific Inc.). Expression of the *Per1* and *Bmal1* targets was normalized to 18S rRNA expression (forward and reverse primer sequences, respectively: 5'-GTC TAA GTA CGC ACG GCC GG-3'; 5'-CAT GCA CCA CCA

CCC ACG GA-3') and calculated by comparison to a relative standard curve created from pooled samples of *Phodopus sungorus* cDNA in serial dilution (1:1, 1:10, 1:100, 1:1000, 1:10,000). Duplicate negative control wells were filled with of 1x Power SYBR Green PCR Master Mix (Life Technologies, Thermo Fisher Scientific Inc.) and the required forward/reverse primers, without cDNA template.

One-way ANOVA analyses were used to determine if statistical significance ($p < .05$) existed between all experimental conditions. Tukey's post-hoc tests were performed to determine which groups were statistically different. Univariate ANOVA tests were utilized to analyze each week's data to determine in which week experimental groups diverged in mass or pelage. Statistical outliers were determined to have a z-score $\geq \pm 2.0$ and were removed from the data set before analysis. Animals were considered outliers if they were non-responders to treatment or fell sick during the experiment.

## Results

### *The Effect of Dim Light at Night (dLAN) on Body Mass*

Total body mass was statistically significant between groups following ten weeks of exposure ($F_{3,178} = 72.557$, $p < 0.01$). Tukey's post-hoc test revealed the SD experimental condition to be significantly different from the LD ($p < 0.01$), SD ($p < 0.01$), and SDdim ($p < 0.01$) conditions. There were no statistical differences in final body mass between the LD, LDdim, and SDdim groups ($p > 0.05$). The SD group first began to differ ($p < 0.05$) from the dLAN groups after 3 weeks and the SD conditions animals differed in body mass ($p < 0.01$) from all conditions following 6 weeks of exposure.

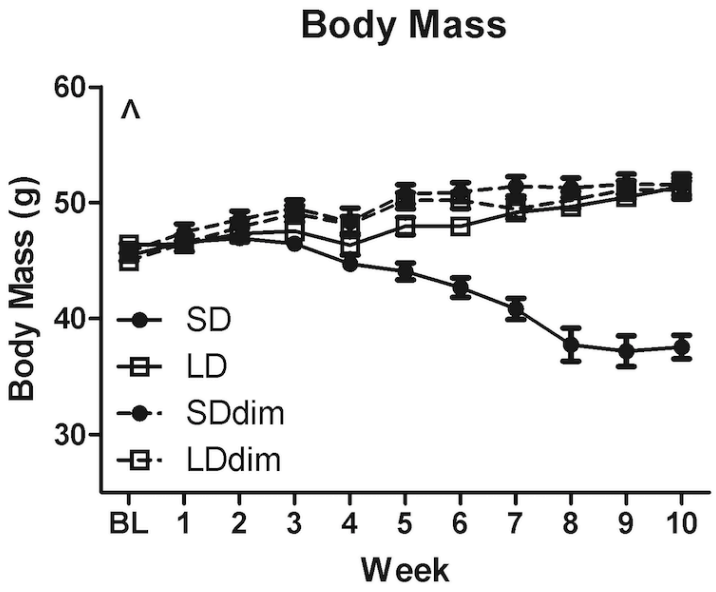


*Fig*. *2*. Average Body Mass over Time (week)

*The Effect of Dim Light at Night (dLAN) on Pelage*

The SD experimental group displayed statistically ($F_{3,174} = 150.954$, $p < 0.01$) lighter colored pelage—a qualitative measure of coat color—after ten weeks of light exposure. Tukey's post-hoc test revealed the SD experimental condition to be significantly different from the LD ($p < 0.01$), SD ($p < 0.01$), and SDdim ($p < 0.01$) conditions. There were no statistical differences in final body pelage between the LD, LDdim, and SDdim groups ($p > 0.05$). The SD group, when compared to the LD group, first began to diverge in pelage coloration ($p < 0.01$) after 4 weeks. The SD condition animals differed in pelage ($p < 0.01$) from all conditions following 7 weeks of exposure.

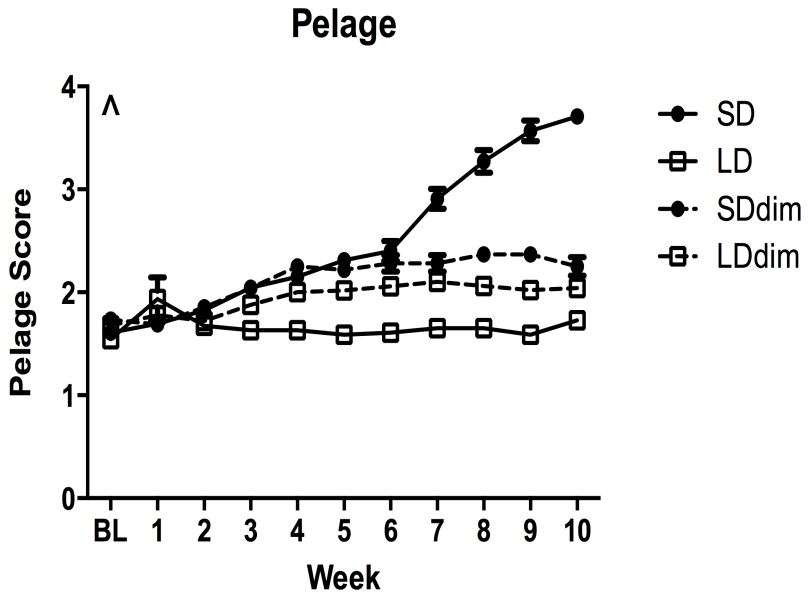


*Fig. 3*. Average Body Pelage over Time (week)

*The Effect of Dim Light at Night (dLAN) on Reproductive Organ Mass*

The SD experimental group was shown to have significantly different reproductive tissue masses from the LD, LDdim, and SDdim groups. The average mass of the testes ($F_{3,174}$ = 470.607, $p < 0.01$), epididymides ($F_{3,178} = 65.466$, $p < 0.01$), seminal vesicles ($F_{3,177} = 19.934$, $p < 0.01$), and gonadal fat pads ($F_{3,158} = 91.453$, $p < 0.01$) at time of euthanasia were all shown to be statistically significant between groups. Tukey's post-hoc test revealed the SD experimental condition reproductive tissue masses to be significantly different from the LD ($p < 0.01$), SD ($p < 0.01$), and SDdim ($p < 0.01$) conditions for the gonadal fat pads, epididymides, testes, and seminal vesicles. There were no statistical differences in gonadal fat pad, epididymides, and seminal vesicle mass between the LD, LDdim, and SDdim groups ($p > 0.05$). There were no statistical differences in testes mass between the LD and LDdim groups ($p > 0.05$).

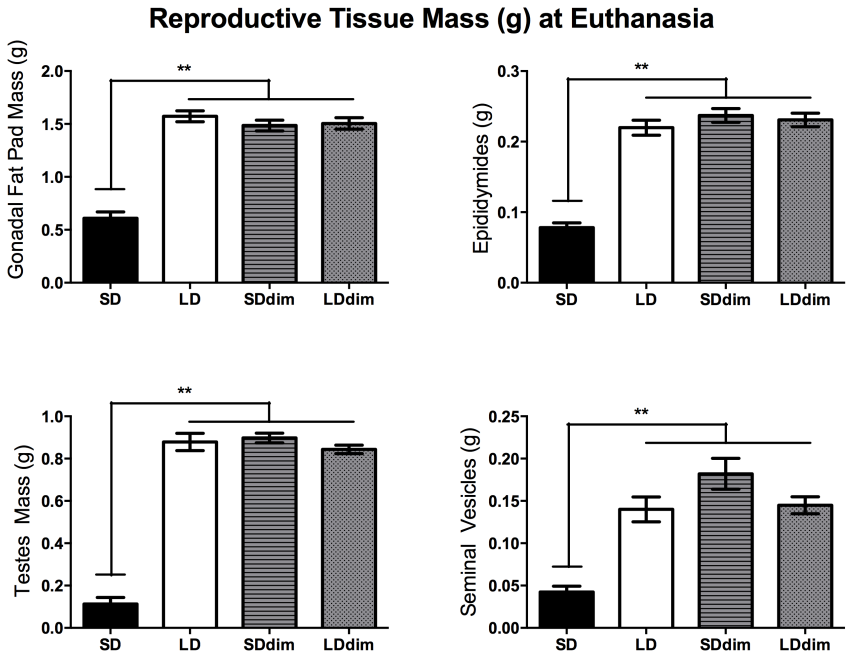


*Fig. 4*. Final Reproductive Tissue Masses (g) at Time of Euthanasia

*qPCR Results for Per1 and Bmal1*

mRNA expression ratios were created for *Per1:*18S and Bmal1:18S to compare relative gene expression. The SD and LD conditions acted as the controls for *Per1* and *Bmal1* expression and must be compared to their dLAN counterparts, SDdim and LDdim, respectively to analyze results. When dLAN was introduced, the expected antiphasic relationship between *Per1* and *Bmal1* was distorted. This is especially clear in *Fig. 4*.; although the LD animals had a clear antiphasic *Per1* and *Bmal1* relationship, the dLAN condition (LDdim) had less clear relationships, especially in the onset of 150-lux light from 2100 to 1300.

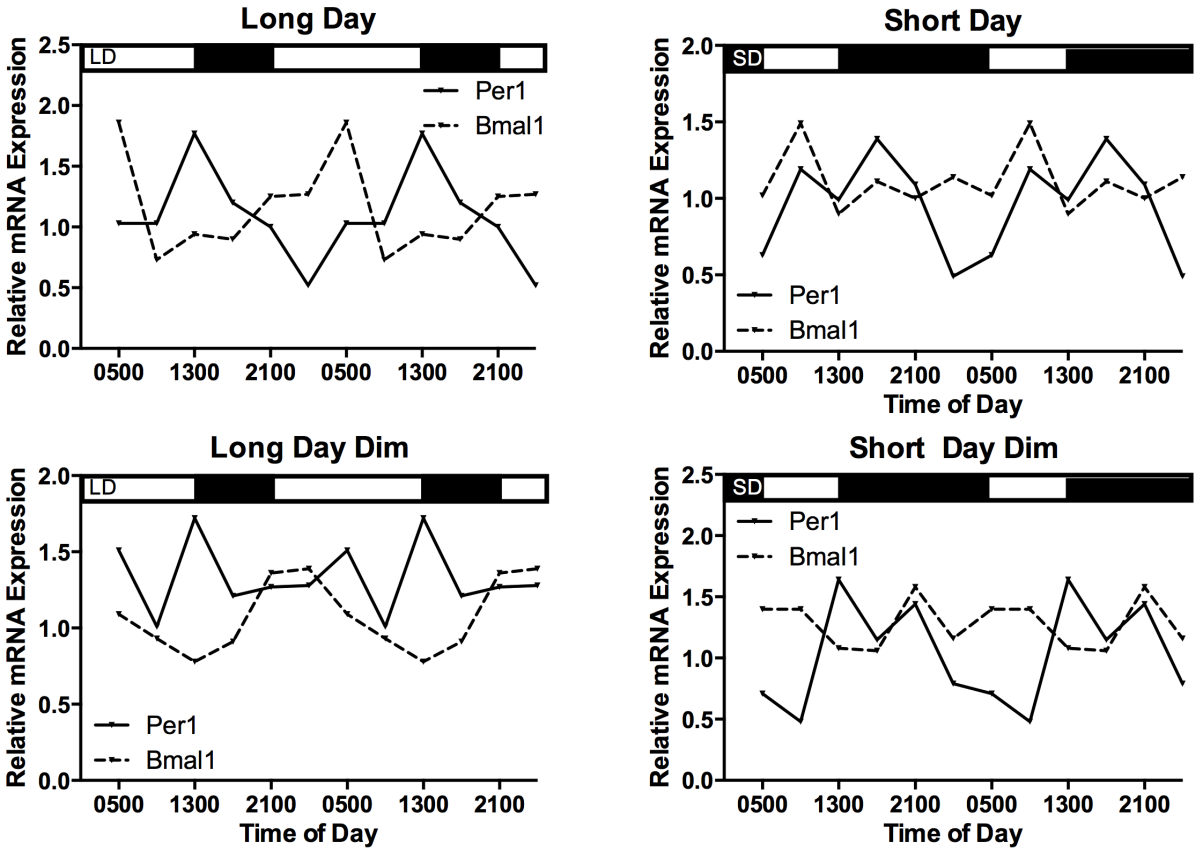


*Fig. 4.* Clock Gene Expression (double-plotted) with and without dLAN

According to *Fig. 5.*, dLAN appears to have shifted the phase of *Per1* expression to the right in the SD/SDdim conditions. Additionally, dLAN has created an antiphasic relationship in otherwise similar *Per1* expression for the LD/LDdim groups between the 1700 and 900 times.

With respect to *Bmal1*, dLAN has created an antiphasic relationship in expression between the SD/SDdim conditions. The graphs in *Fig. 5.* are clearly distorted with the introduction of dLAN for the SD/SDdim conditions. dLAN, however, seems to have had little or no effect on *Bmal1* expression in the LD/LDdim conditions, with the only significant alteration occuring at the 500 time point.

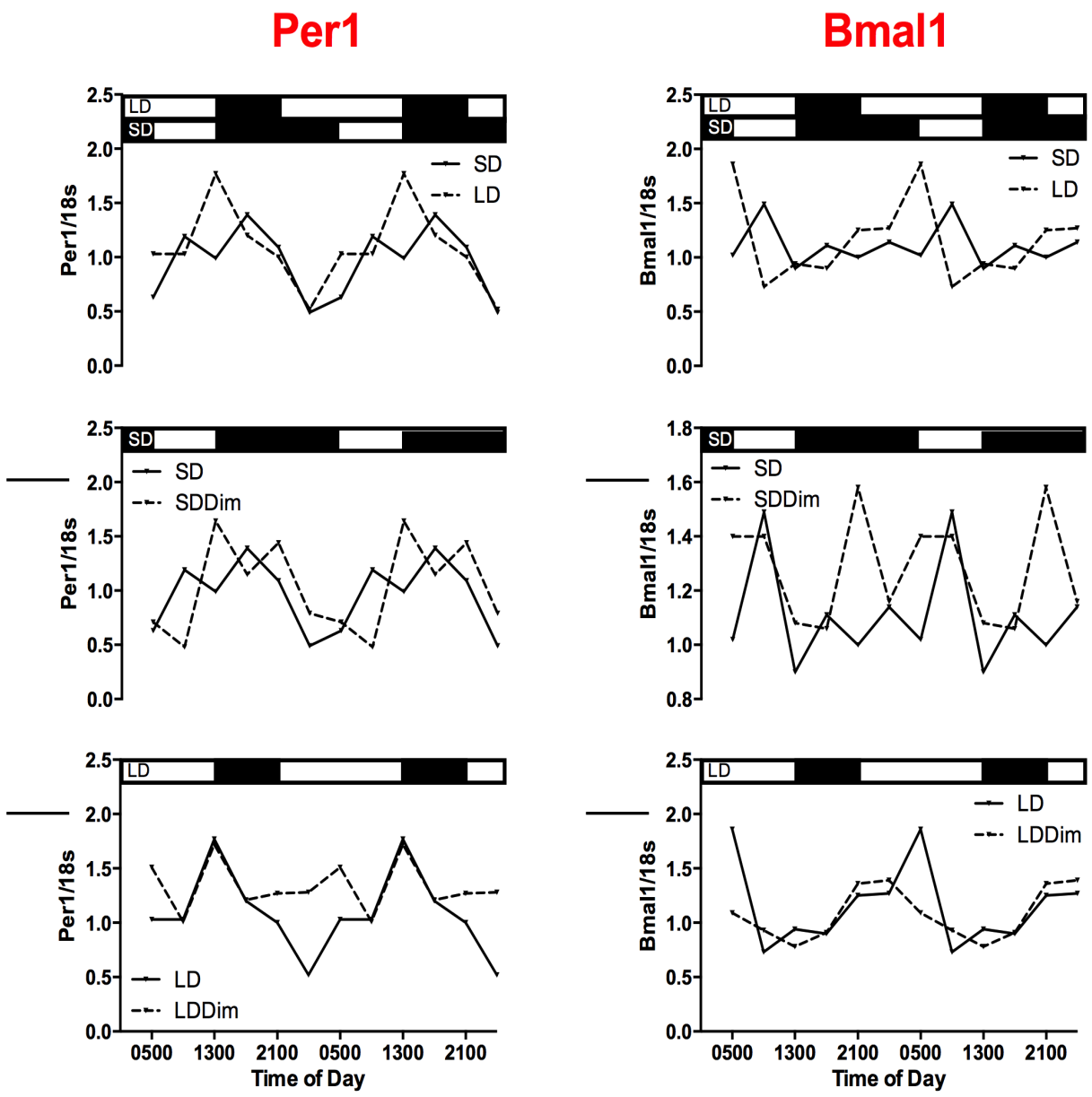


*Fig. 5. Per1/18S* and *Bmal1/18s* Expression Ratios

**Discussion**

Differences in body mass, pelage, and reproductive organ masses were observed between the normal SD phenotype and the LD condition. The SD animals displayed lower body and reproductive organ mass, as well as the lighter, 'winter-like' pelage. The LD animals displayed higher body and reproductive organ mass, as well as the dark, 'summer-like' pelage. These changes were to be expected and are a positive confirmation of a photoperiodic effect on the reproductive system, suggesting a reproductive shutdown in SD animals.

dLAN interfered with the expected reproductive shutdown. The SDdim, LDdim, and LD conditions were not statistically different in body mass, pelage, or reproductive organ mass. As such, the dLAN response was more similar to the LD phenotype response than the SD response. This may suggest that dLAN prevents the reproductive shutdown in non-tropical rodents displaying photoperiodic sensitivity.

The predicted alteration in peripheral immune organ gene expression also occurred with the introduction of dLAN. The presence of light at night muddles the clear distinction between light and dark cycles, perhaps contributing to the differences in gene expression in *Per1* and *Bmal1*. At this time, it is difficult to quantify these differences, as the lymph nodes were pooled to yield greater amounts of RNA to be transcribed to cDNA. However, we can qualitatively tell that dLAN has had an effect on the 'normal' expression of circadian clock genes, perhaps leading to the lack of reproductive shutdown in dLAN animals. Data indicated reproductive tissue masses to be no different in dLAN animals from active reproductive organs in the LD animals. These data suggest that dLAN alters the inherent ability to regulate circadian rhythms and creates alterations in gene expression that can perhaps be linked immune system function with future research.

*Future Directions*

Splenic tissue was also extracted during the euthanasia period for future analysis. At this time, qPCR data collection for *Per1* and *Bmal1* expression is ongoing. Unlike the lymph node tissue, spleen samples were not pooled due to higher expected RNA extraction yields. As such, splenic tissue qPCR data will have greater statistical power than the lymphatic data and can support or deny the lymphatic findings for *Per1* and *Bmal1* expression. Additional clock genes, such as *Clock* or *Per2* may be analyzed, as the influence of dLAN is not yet documented in these genes.

The intention of this study is to find a connection between these alterations in gene expression and immune function. Changes in reproductive tissue masses can suggest a shift in energy investment from the reproductive system to the immune system, but better analyses can be performed to make a clear connection. An enzyme-linked immunosorbent assay (ELISA) is desired to analyze cultured spleen and lymph node supernatants collected in response to LPS following euthanasia for cytokine markers of inflammation. It is expected that the dLAN and LD conditions will have higher levels of inflammation markers than the SD condition, as short photoperiods have been shown to reduce fever, cytokine expression, and 'sickness behavior' when artificially induced by an endotoxin, lipopolysaccharide (LPS). At this time, three different ELISA analyses have been utilized to no success. ELISA analyses are manufactured for use in specific species, but no ELISA kit currently exists for *Phodopus sungorus*.

*Broader Implications*

In an ever-more technologically driven world, dLAN is omnipresent. Light pollution and heavy use of electronics like smartphones, tablets, and computers may have great effects on natural gene expression and immune function. The natural interplay between neuroendocrine and immune system function is being obscured by dLAN, leaving implications for normal photoperiodic function. Future photoperiodic studies may yield greater understanding of the complex neuroendocrine and immune system interplay.

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